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Mutational Evidence for Identity of Penicillin-Binding Protein 5 in *Escherichia coli* with the Major D-Alanine Carboxypeptidase IA Activity

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The defect in D-alanine carboxypeptidase IA activity in the *dacA*11191 mutant of *Escherichia coli* was correlated with a defect in the release of penicillin G from penicillin-binding protein 5. The results suggest that penicillin-binding protein 5 catalyzes the major D-alanine carboxypeptidase IA activity of the wild type and that the mutation results in a defect in the deacylation step catalyzed by this enzyme.

Purified D-alanine carboxypeptidase IA in *Escherichia coli* consists of two polypeptides which can be separated by sodium dodecyl sulfate-acrylamide gel electrophoresis (12). Both proteins bind penicillin G and have been identified as penicillin-binding proteins (PBP) 5 and 6 of *E. coli* (10; see also 9). Both proteins also release the bound penicillin G (8); these abilities represent weak β-lactamase activities (12). Tamura et al. (12) and Curtis and Strominger (1) demonstrated that both activities, i.e., D-alanine carboxypeptidase and release of enzyme-bound penicillin G, are sensitive to sulfhydryl reagents such as *p*-chloromercuribenzoate (pCMB). These reagents have no effect on penicillin G binding and, correspondingly, pCMB causes accumulation of an acyl enzyme intermediate of the carboxypeptidase reaction derived from a synthetic substrate, diacetyl-L-lysyl-D-alanyl-D-alanine (1). The same intermediate was subsequently obtained in higher yield with the more reactive ester substrate diacetyl-L-lysyl-D-alanyl-D-lactate (7). Thus, it was proposed (1, 12) that the binding of penicillin G to D-alanine carboxypeptidase IA and its release correspond to substeps of the total D-alanine carboxypeptidase reaction. The first step (step 1), which is rather insensitive to pCMB, is the formation of an acyl enzyme intermediate with concomitant release of the terminal D-alanine of a substrate such as diacetyl-L-lysyl-D-alanyl-D-alanine or UDP-*N*-acetylmuramyl-pentapeptide. The second step (step 2), which is sensitive to pCMB, is the transfer of the acyl group to water. If the substrate is penicillin, the sum of steps 1 and 2 is the β-lactamase reaction. This paper describes mutational evidence for the identity of these two enzymatic reactions and also suggests the possibility that only PBP 5 is responsible for the enzymatic activity, D-alanine carboxypeptidase IA.

The *dacA*11191 mutant of *E. coli* strain JE11191 (F⁻ *thr leu trp his thyA argH thi lacY malA mtl mel tonA rpsL dacA*), which lacks D-alanine carboxypeptidase IA activity, was described previously (4). Isogenic strains containing either the *dacA*⁺ or the *dacA* allele were obtained by transduction of strain TMRL-122 (F⁻ *proA purB his thi lacY galK rpsL leuS dacB*) with phage P-1 grown in the *leuS*⁺ *dacA*11191 strain. *leuS*⁺ transductants were selected. Strain TMRL-122 was obtained by *argG*⁺ transduction of strain TMRL-12 (F⁻ *proA purB his argG thi lacY galK rpsL leuS*) with phage P-1 grown in an *argG*⁺ *dacB* strain that lacks D-alanine carboxypeptidase IB activity (5). The presence of the *dacB* mutation greatly facilitates the determination of D-alanine carboxypeptidase IA activity. The map positions of the *dacA* and *dacB* genes and the method used for preparation of strains with *dacA dacB* double mutations will be described elsewhere (4, 10a; Y. Hirota, Y. Nishimura, Y. Takagaki, I. N. Maruyama, and M. Matsuhashi, in preparation).

The defect of D-alanine carboxypeptidase activities in these *dacA dacB* strains was demonstrated by the two different assay methods previously described (Table 1): experiment 1, the exchange reaction of [^{14}C]glycine with terminal D-alanine of UDP-MurNAc-pentapeptide (L-

TABLE 1. *Enzyme activities in isogenic dacA⁺ dacB and dacA dacB strains*[a]

Expt	Strain and genotype	Addition	Enzyme activity (pmol/mg of protein per h)
1	TMRL-1224 (*dacA⁺ dacB*)	None	171
		0.1 mM pCMB	19
		0.1 mM pCMB plus 1 mM 2-mercaptoethanol	166
	TMRL-1222 (*dacA dacB*)	None	1
2	TMRL-1224 (*dacA⁺ dacB*)	None	826
		3 μg of penicillin G per ml	540
		10 mM $MgCl_2$	470
	TMRL-1222 (*dacA dacB*)	None	12

[a] D-Alanine carboxypeptidase activity was assayed in an extract [0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.5] of a sonic lysate of late-log-phase cells that had been cultured in L-broth (3) supplemented with 20 μg of thymine per ml. For [^{14}C]glycine exchange (pseudotranspeptidase reaction, experiment 1), the reaction mixture contained in a final volume of 30 μl: 2 μmol of Tris-hydrochloride buffer, pH 9.0; 0.1 μmol of UDP-MurNAc-L-Ala-D-Glu-*m*-A_2pm-D-Ala-D-Ala; 1 nmol of [^{14}C]glycine (102 μCi/μmol, New England Nuclear); sonic cell extract (50 to 100 μg of protein); 0.03 μmol of 2-mercaptoethanol; and 0.67% (wt/vol) Triton X-100. It was incubated for 60 min at 30°C, heated at 100°C for 1 min, and chromatographed on Whatman no. 3 MM filter paper in the solvent isobutyric acid–1 M ammonia (5:3, vol/vol). After chromatography, the radioactive area on the paper was detected in a spark chamber (Birchover Co., England), and radioactivity on the paper corresponding to the product was counted in a liquid scintillation spectrometer by immersing the paper in toluene–PPO (2,5-diphenyloxazole)–POPOP [2,2′-*p*-phenylene-bis-(5-phenyloxazole] (1 liter:4 g:100 mg). For assay of D-[^{14}C]alanine release (experiment 2), the reaction mixture contained, in a final volume of 30 μl: 3 μmol of Tris-hydrochloride buffer (pH 9.0); 0.3 nmol of UDP-MurNAc-L-Ala-D-Glu-*m*-A_2pm-D-[^{14}C]Ala-D-[^{14}C]Ala (20 μCi/μmol, prepared as described previously [2]); sonic cell extract (50 to 100 μg of protein); 0.05 μmol of 2-mercaptoethanol; and 1% (wt/vol, final) Triton X-100. The reaction was carried out for 60 min at 30°C. Subsequent procedures were as described above. Results on other isogenic strains (TMRL-12211 and TMRL-1228, etc.) were similar.

Ala-D-Glu-*m*-A_2pm-D-Ala-D-Ala) (4–6, 12); and experiment 2, release of terminal D-[^{14}C]alanine from UDP-MurNAc-pentapeptide labeled in the terminal D-alanyl-D-alanine group, D-[^{14}C]Ala-D-[^{14}C]Ala (2, 4, 5). Strains *dacA dacB* showed scarcely any activity in either enzyme assay (Table 1). *dacA⁺ dacB* strains showed appreciable activity in both reactions, and this activity was due to D-alanine carboxypeptidase IA activity, as indicated by its intermediate sensitivity to penicillin G, slight inhibition by magnesium ion, inhibition by 100 μM pCMB, and protection from pCMB inhibition by excess 2-mercaptoethanol.

The isogenic *dacA⁺* and *dacA* strains (both containing the *dacB* mutation) gave similar patterns of PBPs. The differences in the amounts of PBP 5 and PBP 6 in the *dacA* mutant strain JE11191 compared to the amounts in the parent strain PA3092 observed previously (4) were not observed in the isogenic *dacA⁺* and *dacA* strains. Therefore, the changes in the amounts of PBP 5 and 6 are not due to the *dacA* mutation itself, but rather to some other genetic or physiological changes occurring in the mutant JE11191.

Curtis and Strominger previously suggested (1; see also 4) that the *dacA*11191 mutation may produce a defect in carboxypeptidase IA similar to the alteration produced by sulfhydryl reagents, which inhibits step 2 of the enzyme reaction. Thus, release of [^{14}C]penicillin G from PBP 5 and 6 was examined in isogenic *dacA⁺ dacB* and *dacA dacB* strains. The release of [^{14}C]penicillin G from PBP 5 was almost completely blocked in *dacA* strains, whereas the release from PBP 6, which is normally much slower than that from wild-type PBP 5, was unchanged in *dacA* strains (Fig. 1). The results were confirmed with 12 *dacA⁺ dacB* and 14 *dacA dacB* strains and with 7 *dacA⁺ dacB⁺* and 5 *dacA dacB⁺* strains (total, 38 strains). The 12 strains containing the *dacB⁺* gene consisted of 10 isogenic strains isolated by a method similar to that described above, the original *dacA* mutant strain JE11191, and its *dacA⁺* parent strain, PA3092.

The following conclusions are drawn from these results: (i) the *dacA*11191 mutation, located at 13.7 min on the *E. coli* chromosome, causes a defect in the release of bound penicillin G from PBP 5; (ii) PBP 5 may be responsible for the major enzyme activity of D-alanine carboxypeptidase IA; and (iii) the penicillin G-releasing activity of PBP 5 appears to be identical to step 2 of the D-alanine carboxypeptidase reaction, in which the acyl enzyme intermediate derived from substrate (the terminal D-alanine having been removed) is transferred to water or another acceptor.

Unlike PBP 5, PBP 6 does not seem to be responsible for the major D-alanine carboxypeptidase reaction, because this protein in *dacA*11191 mutant cells can bind [^{14}C]penicillin G and release it even though the crude cell extract of the mutants lacks D-alanine carboxy-

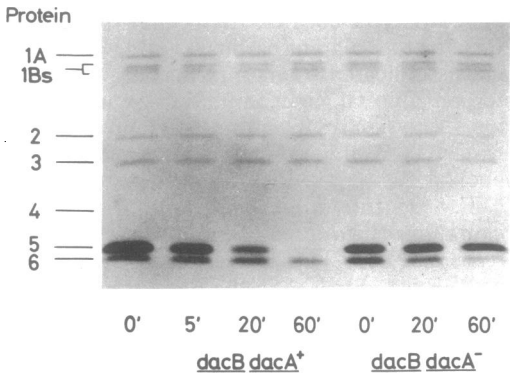

FIG. 1. *Defect in the release of [^{14}C]penicillin G from PBP 5 in the dacA11191 mutant. Release of penicillin from PBP 5 and PBP 6 in the isogenic strains (A) TMRL-12211 (dacA$^+$ dacB) and (B) TMRL-1228 (dacA dacB) is shown. Experimental procedures were essentially as described previously (8–11). Cells in the log phase (about 10^8 cells per ml), grown at 30°C in L-broth (3) supplemented with 20 μg of thymine per ml, were sonically treated in 0.01 M sodium phosphate buffer, pH 7.0, and the membrane fraction was obtained by fractional centrifugation between 3,000 × g (10 min) and 100,000 × g (30 min). The fraction was washed with the same buffer and suspended in 0.05 M sodium phosphate buffer, pH 7.0 (protein concentration, 20 mg/ml). For binding of [^{14}C]penicillin G, a mixture of 30 μl of membrane suspension and 3 μl of [^{14}C]penicillin G (50 μCi/μmol, Radiochemical Centre, Ammersham, England; 1 mM) was incubated for 10 min at 30°C. After binding of [^{14}C]penicillin G to the proteins as described above, 0.9 mg of unlabeled penicillin G (Takeda Chemical Industry Co., Osaka; 800-fold excess) was added to the mixture, and the incubation was continued at 30°C for the indicated periods. Then the reaction mixture was mixed with 2 μl of a solution containing 90 μg of unlabeled penicillin G and 15% (wt/vol) Sarkosyl (Ciba Geigy). The Sarkosyl-insoluble outer membrane fraction was removed by centrifugation at 12,000 × g for 30 min at 20°C, and the supernatant was mixed with 20 μl of a mixture containing 2.25% (wt/vol) sodium dodecyl sulfate, 0.15 M tris(hydroxymethyl)aminomethane - hydrochloride buffer (pH 6.8), 22.5% (wt/vol) glycerol, 0.0015% (wt/vol) bromophenol blue, and 25% (wt/vol) 2-mercaptoethanol and heated for 2 min at 100°C. Then it was subjected to sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. A separating gel containing 7.5% (wt/vol) acrylamide with 1.3% (wt/wt) cross-linking was used (11). The fluorogram of the slab gel was prepared as described previously (8, 11). Results on other isogenic strains (e.g., TMRL-1224 and TMRL-1222) were similar.*

peptidase activity. Further work involving separation of PBP 6 free from PBP 5 is, however, necessary for the identification of the enzymatic activity of PBP 6.

It is possible that the gene for D-alanine carboxypeptidase IA (*dacA*) and the gene for PBP 5 are located very close together on the *E. coli* chromosome and that the first *dacA* mutant isolated, strain JE11191, had independent mutations of both of these genes. It seems very unlikely that the two properties of *dacA* are due to a double mutation, since 36 transductants, 18 of which were *dacA*$^+$ and 18 of which were *dacA*, were isolated. These transductants were selected for the *leuS*$^+$ marker, which is 90% linked to the *dacA* marker by cotransduction with phage P-1. Therefore, the finding that in each of the 36 transductants either both activities were defective or both were normal indicates that these two enzymatic defects are due to a single mutation. The recent observation of Tamaki et al. (10a) that the *dacA* mutation results in supersensitivity of *E. coli* cells to various β-lactam antibiotics indicates that the *dacA*$^+$ revertant can be obtained from *dacA* cells by selecting cells that are less sensitive to β-lactam antibiotics.

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